Online Appendix for the following April 6, 2010 *JACC* article

TITLE: Adenylyl Cyclase 6 Deletion Reduces LV Hypertrophy, Dilation, Dysfunction and Fibrosis in Pressure-overloaded Female Mice

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APPENDIX

METHODS

LV Physiological Study. A 1.4F conductance-micromanometer catheter was used to measure LV hemodynamics using a closed chest method, as previously reported (1).

For the LV physiological study, we used pentobarbital in a higher amount than has been used in previous studies (2). Indeed, 100 mg/kg (intraperitoneal) pentobarbital was required to obtain a deep level of anesthesia, since many animals were semiconscious at lower doses. The LV pressure, obtained during measurement of the pressure-volume relationship, reflects the cardiodepressant effect of pentobarbital. Indeed, 1-2 days prior to the terminal study from which the LV pressure was assessed, we examined transaortic flow rates by Doppler echocardiography. In these studies, we used 1% isoflurane, which is associated with far less negative inotropic effects compared to pentobarbital. These studies documented that blood flow velocity exceeded 4 m/s in almost all cases, indicating a gradient of >64 mm Hg and a substantial pressure load. Finally, LV-to-tibial-length ratios were increased by 60%, which is a similar degree of

LV hypertrophy reported 3 weeks after TAC by others, thereby documenting similar pressure stress (3).

Quantitative RT-PCR. Total RNA was extracted from mouse LV samples using RNA STAT-60 (Tel-Test Inc., Friendswood, TX), treated with RNase-free DNase to eliminate genomic DNA contamination, and purified with RNeasy Mini kit (Qiagen, Valencia, CA). cDNA was synthesized from 4 μg total RNA by reverse transcription reaction using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamers (Invitrogen). The primer pairs used for quantitative RT-PCR analysis of adenylyl cyclase (AC) isoforms (AC2, 3, 4, 5, 7, and 9) were as previously reported (4). Other primer pairs used were:

ANF: forward 5'-CCTCGTCTTGGCCTTTTGG-3'

reverse 5'-CATCTTCTACCGGCATCTTC-3'

α-SK actin: forward 5'-GTGTCACCCACAACGTGC-3'

reverse 5'-AGGGCCACATAGCACAGC-3'

β-MHC: forward 5'-GCTGAAAGCAGAAAGAGATTATC-3'

reverse 5'-TGGAGTTCTTCTCTTCTGGAG-3'

collagen I α1 chain: forward 5'-GCCAAGAAGACATCCCTGAAG-3'

reverse 5'-GGGTCCCTCGACTCCTAC-3'

collagen III α1 chain: forward 5'-GCACAGCAGTCCAACGTAGA-3'

reverse 5'-TCTCCAAATGGGATCTCTGG-3';

FHL1: forward 5'-TGCAACAAGTGCGCTACTCG-3'

reverse 5'-CAATGTTTGGCGAACTTGGTC-3',

periostin: forward 5'-CAGCGGGTCATGGCTAAC-3'

reverse 5'-CAGCTTGCCATCTTGGAGTC-3'

Quantitative real-time PCR was conducted on a Mx3000P QPCR System (Stratagene, La Jolla, CA) using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) under the following conditions: 5 min at 98°C, 40 cycles of 30 s at 95°C, 30s at 55°C, and 30s at 72°C. RNA equivalents were normalized to simultaneously determined glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels in each sample. Relative RNA in LV samples from AC6-KO mice was compared to that from CON mice. Specificity of each RT-PCR reaction was checked by its dissociation curve. Single product amplification and correct product size were confirmed by agarose gel electrophoresis.

For quantitative RT-PCR using RNA from neonatal rats, the primer pairs were used:

AC6: forward 5'-GTCTTTCCACCCTGCATTG-3'

reverse 5'-GTAACCACGGGTCTCCTGAA-3'

ANF: forward 5'- GTATACAGTGCGGTGTCCAAC-3'

reverse 5'- GAGAGCACCTCCATCTCTC-3'

β-MHC: forward 5'-GCTGAAAGCAGAAAGAGATTATC-3'

reverse 5'-TGGAGTTCTTCTCTTCTGGAG-3'

collagen I α1 chain: forward 5'-GCCAAGAAGACATCCCTGAAG-3'

reverse 5'-CTTCTGGGCAGAAAGGACAG-3'

collagen III α1 chain: forward 5'-GGTTTCTTCTCACCCTGCTTC-3'

reverse 5'-TCTCCAAATGGGATCTCTGG-3';

Western Blotting. LV samples were homogenized at 4°C in homogenization buffer (25 mmol/L Tris-HCl, pH 7.4, 0.5 mmol/L EDTA, 0.5 mmol/L EGTA, 50 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4) in the presence of protease inhibitor cocktail (Roche, Indianapolis, IN). Samples were denatured in Laemmli buffer and subjected to SDS-PAGE. After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA), which were incubated in blocking buffer (5% milk, 20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.1% Tween 20) for 1 h and then incubated overnight with the antibodies for FHL1 (four and a half LIM domains 1; Cat # EB06507, Everest Biotech Ltd., Oxfordshire, UK), periostin (Cat # AF2955, R&D Systems, Minneapolis, MN), PDE3A (Cat # sc-20792, Santa Cruz Biotechnology, Santa Cruz, CA), PDE4 (Cat # PD4-101AP, Frisco, TX). Binding of the primary antibody was detected by an enhanced chemiluminescence method (ECL^+, Amersham Biosciences, Piscataway, NJ) using horseradish peroxidase-conjugated donkey anti-goat IgG or goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Reprobing stripped blots with GAPDH antibody enabled evaluation of equal loading and transfer efficiency. Quantification of protein expression was performed using Gel-Pro® Analyzer (Media Cybernetics, Silver Spring, MD).

Gelatin Zymography. LV homogenates were denatured in Laemmli buffer containing no β-mercaptoethanol and used for gelatin zymography (5).

RESULTS

We assessed LV protein contents of PDE3A and PDE4D, two important cAMP-hydrolyzing phosphodiesterases in cardiac myocytes, in CON and AC6-deleted mice 3w after TAC. We found that AC6 deletion did not alter protein contents of PDE3A [CON: 493±152 densitometric units (du), AC6KO: 414±66 du; p=0.64, n=8 for both groups] and PDE4D (CON: 636±67 du, AC6KO: 685±107 du; p=0.70, n=8 for both groups).

We also found that AC6 deletion did not alter mRNA contents of AC isoforms (AC2, 3, 4, 5, 7, and 9) in pressure overloaded hearts (Figure).

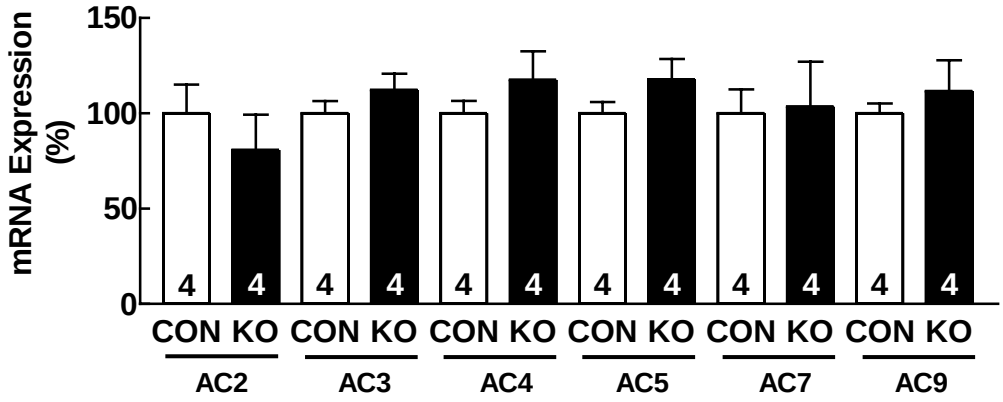

Figure AC6 deletion did not alter mRNA contents of AC2, AC3, AC4, AC5, AC7, and AC9 in pressure overloaded hearts. Error bars denote 1 SE; numbers in bars indicate group size.

To identify the abnormalities associated with early death observed in male AC6-KO mice, we conducted echocardiographic studies on males and females 1w after TAC. The male AC6-deleted mice already show greater LV dilation and decreased function vs the females (Table). Expression of FHL1 and periostin mRNA was also higher in male AC-KO mice than females 1w after TAC.

	Female AC6-KO 1w after TAC	**Male AC6-KO 1w after TAC**	**p**
EDD (mm)	3.2±0.1[A]	4.4±0.5[B]	0.06
ESD (mm)	2.2±0.1[A]	3.8±0.2[B]	0.0001
EF (%)	61±4[A]	26±13[B]	0.04
Vcf (cir/s)	6.1±0.6[C]	2.6±1.4[A]	0.06
LV/TL (mg/mm)	6.1±1.8[A]	8.9±1.4[B]	0.23
ANF	100±25%[C]	119±10%[D]	0.48
α-SK actin	100±19%[C]	130±23%[D]	0.32
β-MHC	100±49%[C]	79±30%[D]	0.71
FHL1	100±15%[C]	160±18%[D]	0.02
periostin	100±33%[C]	187±26%[D]	0.05

EDD, end-diastolic diameter; ESD, end-diastolic dimension; EF, left ventricular ejection fraction; Vcf, velocity of circumferential fiber shortening, LV/TL, left ventricle weight to tibial length ratio. P values are from Student's t test (2-tailed). Entries denote mean±SE. [A]n=10; [B]n=14; [C]n=7; [D]n=8.

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